JOURNAL OF BACTERIOLOGY, Nov. 1980, p. 630–640
0021-9193/80/11-0630/11$02.00/0

Vol. 144, No. 2

Size Heterogeneity of *Salmonella typhimurium* Lipopolysaccharides in Outer Membranes and Culture Supernatant Membrane Fragments

ROBERT S. MUNFORD,[1]* CATHERINE L. HALL,[1] AND PAUL D. RICK[2]

Department of Internal Medicine and Department of Microbiology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235[1] and Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014[2]

Enterobacteriaceae cells growing in liquid media shed fragments of their outer membranes. These fragments, which may constitute a biologically important form of gram-negative bacterial endotoxin, have been reported to contain proteins, phospholipids, and lipopolysaccharides (LPS). In this study we compared the sizes of LPS molecules in shed membrane fragments and outer membranes from cells growing in broth cultures. Using conditional mutants of *Salmonella typhimurium* which incorporate specific sugars into LPS, we analyzed radiolabeled LPS by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This technique revealed that *S. typhimurium* LPS are more heterogeneous than previously known; molecules possessing from 0 to more than 30 O-chain repeat units were identified in outer membranes, supernatant fragments, and purified LPS. The size distributions of LPS molecules in outer membranes and supernatant fragments were similar; supernatant fragments appeared to be slightly enriched in molecules with long O-polysaccharide chains. Our results indicate that LPS molecules of many sizes are synthesized, translocated to outer membranes, and released into culture supernatants. Since the hydrophilic O-polysaccharides extend from bacterial surfaces into the aqueous environment, our findings suggest that the cell surface topography of this bacterium may be very irregular. We also speculate that heterogeneity in the degree of polymerization of O-antigenic side chains may influence the interactions of the toxic moiety of LPS (lipid A) with host constituents.

As *Enterobacteriaceae* cells grow in liquid media, they release membrane fragments (blebs) which have been reported to contain lipopolysaccharides (LPS), phospholipids, and proteins (30). Existing evidence suggests that these fragments are not released randomly from cell surfaces. They seem to be shed at an early stage of outer membrane synthesis (17) and to differ in protein composition from total outer membrane (8, 29). The composition of the blebs and the mechanism by which they are released may thus provide clues to the poorly understood process of outer membrane assembly. These membrane fragments may also be important for a quite different reason; it is possible that they resemble the form in which toxic LPS (endotoxin) are released by gram-negative bacteria growing in vivo. There is no direct evidence to support this idea, yet it seems likely that culture supernatant LPS more closely approximate the chemical and physical properties of "natural" endotoxin than do the LPS which are extracted from these bacteria by hot 45% phenol, trichloroacetic acid, or other standard methods (14).

Other workers have examined the protein, LPS, and phospholipid compositions of supernatant membrane fragments of *Escherichia coli* and *Salmonella* spp. grown under conditions of restricted protein synthesis (12, 29) or using mutant strains which produce defective LPS (8, 12, 17, 29). The present study was undertaken to compare the LPS molecules in supernatant blebs and outer membranes isolated from the same cultures of growing *Salmonella typhimurium*. We used conditional mutants which incorporate specific sugars into their LPS. Intrinsically labeled LPS were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a technique which separates LPS molecules having different polysaccharide contents (9). We found that the LPS made by these strains were unexpectedly heterogeneous in size, and we describe experiments in which this conclusion was validated. The technique was then

used to compare the size distributions of LPS molecules in outer membranes and culture supernatant membrane fragments.

MATERIALS AND METHODS

Bacterial strains and media. Three strains of *S. typhimurium* were provided by M. J. Osborn and H. C. Wu, University of Connecticut Health Center, Farmington. Strain G-30, which is deficient in uridine diphosphate-galactose-4-epimerase, incorporates galactose almost exclusively into LPS (26); galactose is present in both the R-core and O-side chain in *S. typhimurium* (Fig. 1). Strain SL 1030 is deficient in phosphomannose isomerase (28); exogenous mannose is utilized solely for O-chain synthesis in this mutant. Strain SL 2533 is deficient in both uridine diphosphate-galactose-4-epimerase and phosphomannose isomerase, so that its LPS may be labeled with both galactose (in core and O-chain) and mannose (in O-chain). *S. typhimurium* PR 122 is a *galE nag* derivative of *S. typhimurium* SU 453 (*hisF1009 trpB2 metA22 xyl-1 strA201* F⁻) which was isolated by P. Rick by the method of Wu and Wu (32). The *galE* mutation was introduced by P22 transduction, using *S. typhimurium* G-30 as the donor. This strain does not utilize glucosamine for growth. In the absence of exogenous galactose, it incorporates glucosamine into lipid A and into peptidoglycan; when exogenous galactose is present, glucosamine may also be incorporated into the R-core (Fig. 1). This mutant is also deficient in uridine diphosphate-galactose-4-epimerase, so that the R-core and O-chain may be labeled with galactose as described above. Strain SL 1034, an SR mutant which synthesizes LPS that has a single O-chain repeat unit attached to the R-core, was provided by H. Nikaido, University of California, Berkeley. The identities of these strains were confirmed by using their sugar utilization patterns and sensitivities to LPS-specific bacteriophages. *Salmonella minnesota* strains were provided by O. Luderitz, Freiburg, West Germany. Broth cultures of *S. typhimurium* were grown in proteose peptone-beef extract–0.5% NaCl (PPBE) (26) containing supplements as described below.

Radioisotopes. D-[1-^{14}C]mannose (59 mCi/mmol), D-[2-^{3}H]mannose (14.1 Ci/mmol), and D-[1-^{3}H]galactose (14.2 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. *N*-acetyl-D-[1-^{3}H]glucosamine hydrochloride (11 Ci/mmol) and D-[1-^{14}C]-glucosamine hydrochloride (57.9 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill.

LPS preparation. Cells harvested in the late logarithmic phase of growth were extracted with 45% phenol and purified as previously described (21). LPS from *S. minnesota* R-core LPS mutants and from *S. typhimurium* 1034 were prepared by the method of Galanos et al. (6); the *S. minnesota* mutants were grown in Trypticase soy broth (BBL Microbiology Systems).

LPS assay. A solid-phase radioimmunoassay for O-antigen was used (21). The standard was *S. typhimurium* LPS prepared from strain G-30 grown in PPBE containing 0.5 mM D-galactose. This assay measured LPS concentrations over the range from 0.001 to 5.0 μg/ml.

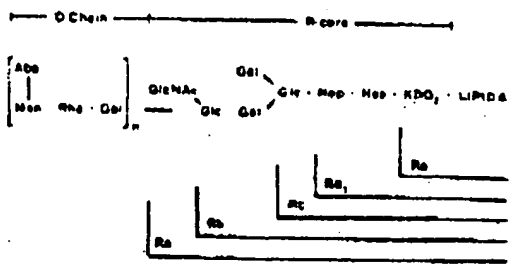

FIG. 1. *Salmonella LPS structure. The structure of S. typhimurium LPS is shown; subscripts indicate the structures of the S. minnesota R-core mutants used for gradient SDS-PAGE (see Fig. 4). Abe, Abequose; Man, mannose; Rha, rhamnose; Gal, galactose; GlcNAc, N-acetylglucosamine; Glc, glucose; Hep, heptose; KDO, ketodeoxyoctonate. The S. minnesota mutant strains used and their phenotypes (in parentheses) were as follows: R60 (Ra), R345 (Rb), R5 (Rc), R7 (Rd1), and R595 (Re). The Rc and Rd1 mutants also lack a phosphate attached to heptose (18). When prepared from cultures grown without galactose, LPS from S. typhimurium strains G-30, SL 3533, and PR 122 have an Rc structure. Each lipid A moiety contains two molecules of glucosamine.*

Growth experiments. To label strain G-30 with radioactive galactose, 100 ml of PPBE containing 0.1 mM D-galactose and 1 mCi of [^{3}H]galactose was inoculated with 2 ml of a growing culture of strain G-30 cells. Cultures were shaken at 150 rpm in a 37°C water bath, and growth was monitored with a Coleman Junior II spectrophotometer by measuring optical density at 540 nm (1.2-cm light path; Perkin-Elmer, Coleman Instruments Div., Oak Brook, Ill.). Cells were harvested after three to four generations of growth (optical density at 540 nm, 0.4), chilled rapidly in an ice water bath, and pelleted by centrifugation at 16,000 × *g* for 5 min at 5°C. Approximately 50% of the [^{3}H]galactose was incorporated into cells under these conditions. Subsequent steps in the preparation of membranes and supernatant blebs were performed at 1 to 4°C.

To label strain SL 3533 with radioactive mannose and galactose, 50 ml of PPBE containing 100 μCi of [^{3}H]galactose, 10 μCi of [^{14}C]mannose, 0.01 mM nonradioactive galactose, and 0.01 mM nonradioactive mannose was inoculated with 2 ml of a growing culture of cells. Higher galactose concentrations inhibited the growth of this strain. Incorporation of mannose into the cells after 3.5 generations of growth was 66%, and incorporation of galactose was 92%.

Strain PR 122 was grown in PPBE containing 0.06 mM D-glucosamine hydrochloride. The cells in 40 ml of medium were labeled with 300 μCi of *N*-acetyl-[^{3}H]glucosamine or 15 μCi of [^{14}C]glucosamine. When desired, 0.06 mM nonradioactive D-galactose was added to permit synthesis of complete LPS; labeling with [^{3}H]galactose was performed as described above. Incorporation of labeled glucosamine into cells after 3.5 generations of growth was 40 to 60%.

Outer membrane separation. Labeled cells were suspended in 2 ml of 25% sucrose in 10 mM HEPES

[4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] buffer (pH 7.4) containing 4 μg of RNase per ml and 4 μg of DNase per ml. After disruption in a French press as described by Jones and Osborn (10), the preparation was brought to final concentrations of 20% sucrose and 5 mM EDTA in 10 mM HEPES. The sample was then layered onto a cushion of 60% sucrose and centrifuged at 100,000 × *g* for 3 h at 6 to 8°C (4). The membrane bands were recovered from the sucrose cushion, resuspended in 10 mM HEPES to a sucrose concentration of 25 to 30%, and layered onto a 30 to 55% sucrose gradient. Ultracentrifugation was for 22 h at 260,000 × *g* and 6 to 8°C. Fractions were collected by puncturing the bottom of the tube, diluted 1:20 or more in distilled water, and tested for protein concentration by absorbance at 280 nm (corrected for absorbance at 260 nm [31]). Radioactivity was determined by adding 25-μl portions of these dilutions to 5 ml of Aquasol (New England Nuclear Corp.) and counting in a Packard model 2425 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). NADH oxidase activity was measured as described by Osborn et al. (26) and was related to protein concentration, which was determined by a modification of the Lowry method (16).

Supernatant LPS. For assays of the LPS concentrations in culture supernatants, 5 ml of culture fluid was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was recentrifuged. The upper 1 ml was removed for assay. This procedure produced a reduction in viable bacterial counts of ≥ 10^4-fold and left fewer than 10^7 viable cells per ml in the supernatant. This number of cells did not interfere with the radioimmunoassay. For analyses of the LPS in supernatant fragments from isotopically labeled cultures, it was necessary to eliminate residual cells from 40 to 60 ml of supernatant by filtration (0.45-μm filter, Sybron/Nalge, Rochester, N.Y.). Cultures of the filtrate were sterile. The concentration of LPS measured in the filtrate by radioimmunoassay was equal to that found in the centrifuged supernatant, indicating that there was no loss of LPS onto the filter. Membrane fragments containing LPS were then precipitated from the filtered supernatant by adding 1.2 volumes of saturated ammonium sulfate (final concentration, 55%). This procedure quantitatively removed LPS from the supernatant and allowed complete recovery of radioimmunoassay activity from the precipitate. The precipitates were suspended in sucrose-HEPES and layered onto sucrose gradients for ultracentrifugation in parallel with membrane fractions, as described above.

SDS-PAGE. Polyacrylamide (6%) tube gels containing 1% SDS were prepared and run by the method of Fairbanks et al. (5), as modified by Jann et al. (9). Samples were solubilized in digestion buffer (9) by heating for 3 min at 100°C. After electrophoresis, gels were cut into 2-mm slices, digested with 30% H_2O_2, and counted as previously described (20). Slab gels contained polyacrylamide (6 to 16.5% linear gradient) and 0.25% high-molecular-weight polyacrylamide (no. 29788; BDH Chemicals, Poole, England); other reagents were as described by Fairbanks et al. (5). A gel overlay (5% polyacrylamide) was added for loading the samples. Slab gels were run at a constant current (10 mA) until the pyronin Y dye just reached the bottom of the gel (20 to 22 h). Staining of gels with periodic acid-Schiff reagent was performed as described by Fairbanks et al. (5), and fluorography was by the method of Bonner and Laskey (3).

RESULTS

Outer membrane preparation. We found that preliminary centrifugation in the presence of EDTA reduced inner membrane and nucleic acid contamination of outer membrane preparations (4). Isolated outer membranes from strain G-30 had an apparent bouyant density of 1.22 to 1.25 g/ml, contained more than 65% of membrane [^{3}H]galactose, and were deficient in NADH oxidase, an inner membrane marker (Fig. 2). These findings are characteristic of outer membranes from *S. typhimurium* (26). Similar results were obtained with strain SL 3533 cells labeled with [^{3}H]galactose and [^{14}C]mannose; the galactose-mannose ratio was constant throughout the gradient (data not shown).

SDS-PAGE analysis of LPS composition. The basis for this approach was the observation of Jann et al. (9) that the migration distance of an LPS in SDS-PAGE is directly related to the percentage of lipid A in the LPS molecule. When we studied [^{3}H]galactose-labeled strain G-30 LPS by using the tube gel system of Jann et al. (9), we found three major peaks of activity (Fig. 3A). We expected (from the work of Jann et al.)

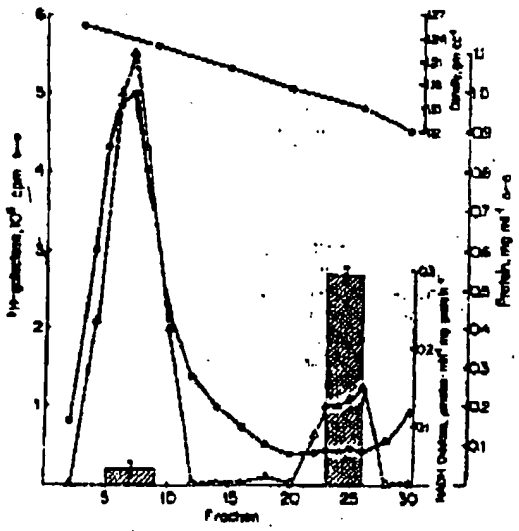

FIG. 2. *Outer membrane preparation. Membranes were prepared and separated by sucrose gradient ultracentrifugation as described in the text. The cross-hatched boxes indicate NADH oxidase activities in fractions pooled from the peaks of protein concentration; bars indicate ± standard deviation.*

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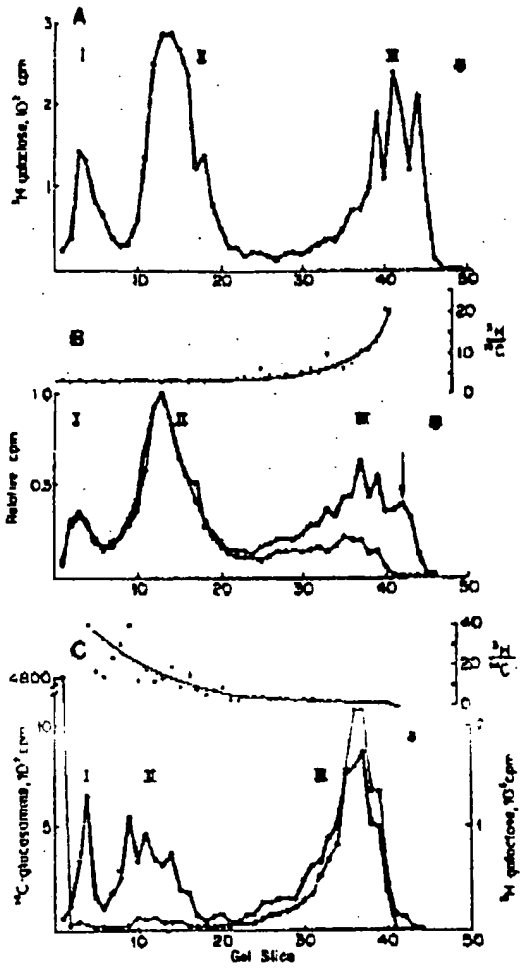


FIG. 3. *(A) Tube SDS-PAGE of [^{3}H]galactose-labeled strain G-30. The top of the gel is on the left. The Roman numerals indicate the three major peaks of radioactivity (for the quantitative comparisons described in the text, peak III included fractions 21 through 47). The arrow shows the pyronin Y dye front. (B) Tube SDS-PAGE of [^{3}H]galactose- and [^{14}C]mannose-labeled strain SL 3533. The ^{3}H and ^{14}C counts per minute (corrected for background and channel spill-over) were normalized to let the counts in gel slice 13 (peak II) equal 1.0. Ratios of ^{3}H to ^{14}C were calculated from the actual corrected counts per minute in each gel slice. The short arrow indicates the dye front. The absence of [^{14}C]mannose (○) from the fastest-migrating peak of [^{3}H]galactose activity (●) indicates that this peak contained only R-core LPS (long arrow). (C) Tube SDS-PAGE of [^{3}H]galactose- and [^{14}C]glucosamine-labeled strain PR 122. Radioactivity measurements were performed as described above for (B). The arrow indicates the dye front. The ^{14}C-labeled material at the origin (top) of the gel probably represents peptidoglycan, since peptidoglycan contains glucosamine and does not migrate into SDS-polyacrylamide gels (20). Symbols: ●, [^{3}H]galactose; ○, [^{14}C]glucosamine. The ratio of ^{3}H to ^{14}C decreased with increasing migration distance (see text).*

that the fastest-migrating LPS would be R-core-lipid A, but the radioactivity profile in the gel was quite irregular, suggesting that more than one LPS species might be present. We then studied strain SL 3533 labeled with both [^{3}H]-galactose and [^{14}C]mannose. Tube SDS-PAGE of double-labeled outer membranes (Fig. 3B) showed that the ratio of ^{3}H to ^{14}C increased with increasing migration distance and that mannose was not present in the fastest-migrating peak. This behavior would be expected if only core LPS were present in the fastest-migrating peak, since core LPS contains galactose but not mannose (Fig. 1). The observed ratios of ^{3}H to ^{14}C along the gel are consistent with the addition of a repeat unit which contains mannose and galactose to a core which contains only galactose. At the top (origin) of the gel, there was little variation in the mannose-galactose ratio, since the long O-side chains minimized the contribution of the galactose-containing core. Finally, we studied the incorporation of glucosamine and galactose in strain PR 122. Tube gel analysis of double-labeled outer membranes (Fig. 3C) showed that glucosamine was present primarily in peak III; since incorporation of glucosamine into LPS is independent of O-chain length, this observation indicates that this strain preferentially makes LPS molecules with relatively short O-chains. The ratio of [^{3}H]galactose to [^{14}C]glucosamine (which provides an estimate of the ratio of O-chain plus R-core to R-core plus lipid A) decreased with increasing migration distance along the gel. This finding again indicates that migration distance in this system is inversely related to LPS polysaccharide content.

These results were confirmed and extended by gradient slab SDS-PAGE. This technique resolved *S. minnesota* core LPS molecules which differed in size by only two to three saccharides (Fig. 4A) and demonstrated a large series of bands for LPS extracted from strain G-30 cells grown in the presence of galactose. The G-30 bands were present on gels stained with periodic acid-Schiff reagent and on fluorographed gels, and they were observed with purified LPS, outer membranes, and supernatant blebs. They did not stain with the Coomassie blue stain for protein. The fastest-migrating G-30 band comigrated with Rc *S. minnesota* LPS; this was not expected (galactose-containing core should be Ra or Rb [Fig. 1]), and the explanation for this result is not known. Differences between

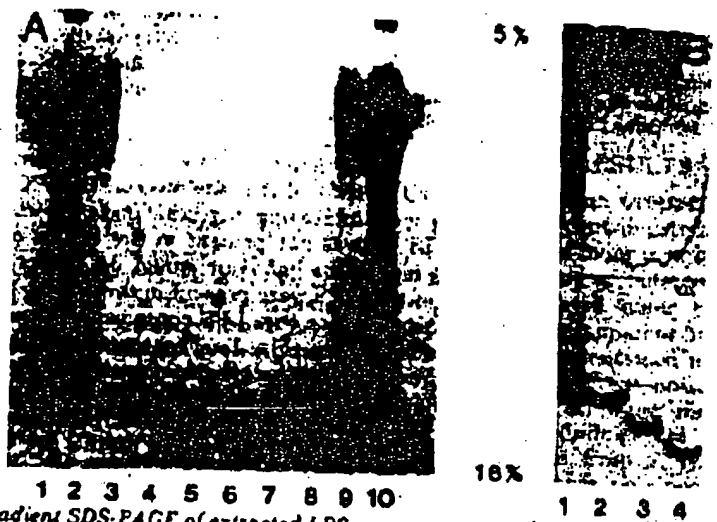


FIG. 4. *(A) Gradient SDS-PAGE of extracted LPS preparations. The gel was stained by the periodic acid-Schiff reagent method. Lanes 1 and 10, Strain G-30 LPS (galactose added to culture) (70 μg/lane); lanes 2 and 9, G-30 LPS (40 μg/lane); lanes 3 through 7, 10 μg of LPS from S. minnesota mutants Ra, Rb, Rc, Rd, and Re, respectively; lane 8, 8 μg each of Ra LPS, Rc LPS, and Re LPS. The concentrations of polyacrylamide in the gradient are shown to the right of the gel. A 5% gel overlay (not shown) contained no periodic acid-Schiff stain-positive material. (B) Gradient SDS-PAGE of extracted LPS preparations. Gel preparation was as described above for (A); electrophoresis time was slightly longer. Lane 1, 100 μg of LPS extracted from a clinical isolate of S. typhimurium; lane 2, 20 μg of LPS from strain SL 1034, an SR mutant; lanes 3 and 4, 10 μg of Ra LPS and 10 μg of Rd LPS, respectively.*

the preparations included the kind of growth medium and the method of LPS extraction (see above). The strain G-30 bands were generally distributed into three populations, corresponding to the three peaks observed with tube SDS-PAGE. These bands were also present in strain SL 3533 LPS double labeled with [^{3}H]galactose and [^{14}C]mannose (Fig. 5); only traces of mannose were present in the fastest-migrating band, as previously noted in the tube gel analysis.

When strain PR 122 was labeled with *N*-acetyl-[^{3}H]glucosamine in the absence of exogenous galactose, almost all of the radioactivity appeared in a single band at the bottom of the gel; this represented labeled Rc LPS (Fig. 6, lanes 1 and 2). A band of radioactivity also remained at the top of the gel, which is consistent with the incorporation of *N*-acetylglucosamine into peptidoglycan (20). In a similar labeling experiment, the addition of nonradioactive galactose allowed the completion of the R-core and the synthesis of O-chains, so that glucosamine-labeled bands appeared which migrated more slowly than the completed R-core (Fig. 6, lane 3). When the same strain was labeled with [^{3}H]galactose instead of glucosamine (Fig. 6, lane 4), the labeling pattern was identical to that found with the *galE* mutants shown in Fig. 7. The findings with gradient SDS-PAGE are thus consistent with the tube gel results. In both gel systems, the distribution of radioactive glucosamine in labeled strain PR 122 indicated that most of the LPS molecules made by this strain have short O-chains (peak III). Similarly, the galactose-glucosamine ratios in peaks I and II provide further evidence that these peaks contain LPS molecules with long O-polysaccharide chains.

The stability of the banding pattern to treatment with SDS at 100°C and the ratios of ^{3}H to ^{14}C observed in tube gel analysis made it unlikely that the multiple bands were artifactually generated by LPS aggregation. We also obtained evidence that the bands did not represent free polysaccharide chains or synthetic intermediates of LPS. First, the polysaccharide produced by mild acid hydrolysis of LPS (9, 21) contains O-chain and R-core sugars, yet this preparation remained at the top (origin) in both gel systems (data not shown). This observation provided further evidence that LPS migration during SDS-PAGE requires that lipid A be present. Second, the multiple bands observed with gradient SDS-PAGE were present in LPS after extraction with hot 45% phenol (Fig. 4, 5, and 7), a treatment which releases O-polysaccharide from the carrier lipid to which it is linked during O-chain subunit polymerization (11). Since polysaccharides do not migrate in these gels without linkage to lipid, it seems unlikely that intermediates in O-chain synthesis could account for the

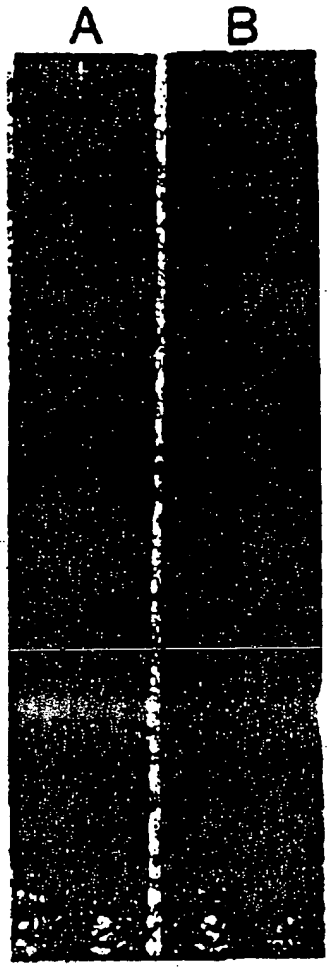


FIG. 5. *SDS-PAGE analysis of strain SL 3533 LPS labeled with [^{3}H]galactose and [^{14}C]mannose. The orientation of the gel was the same as in Fig. 4. The gel was cut into two parts; part A was fluorographed to detect ^{3}H and ^{14}C in the bands, and part B was autoradiographed to detect ^{14}C only. Lanes 1 and 4, Outer membranes of SL 3533 labeled with [^{3}H]galactose and [^{14}C]mannose as described in the text; lanes 2 and 3, [^{14}C]mannose-labeled SL 1030 LPS (phenol-water extract). Note that only [^{3}H]galactose was present in the fastest-migrating band (lane 1).*

multiple bands observed with phenol-extracted preparations. Finally, the multiple bands were also observed when strain G-30 cells were grown with [^{3}H]galactose for one generation, followed by two generations of growth with a 20-fold excess (1 mM) of nonradioactive galactose. Failure of the bands to "chase" into LPS with longer O-chains is again evidence that the banding pattern represented completed LPS.

As shown above, the fastest-migrating labeled species was core LPS. The regularity of the banding pattern and the degree of resolution demonstrated in the core region made it likely that each band on the gradient gel contained LPS molecules which differed in size from those in adjacent bands by one O-chain repeat unit. This conclusion was confirmed by using *S. typhimurium* SL 1034, a mutant which attaches only one O-chain repeat unit to core LPS. LPS extracted from SL 1034 cells produced only one band of periodic acid-Schiff stain-positive material, which was in a gel position that corresponded to the position of LPS containing a single O-chain repeat unit (Fig. 4B). Thus, it was possible to determine from the bands in the gradient gels that tube gel peak III contained LPS with 0 to 16 repeat units and to estimate (by extrapolation) that the average number of repeat units in peak II LPS was 28 to 30.

Although the evidence presented above suggests strongly that peak I contained LPS molecules with very long O-chains (constant mannose-galactose ratio, high galactose-glucosamine ratio, slow migration rate), the number of repeat units present in peak I LPS could not be estimated accurately from our gels. The distribution of LPS into peaks I and II was somewhat variable from experiment to experiment, even when the same medium and bacterial strain were used.

Multiple periodic acid-Schiff reagent-stained bands were also found in LPS extracted from clinical isolates of *S. typhimurium* (Fig. 4B), *Salmonella typhi*, and *E. coli* (data not shown), indicating that LPS size heterogeneity is not limited to the conditional mutants used for these experiments.

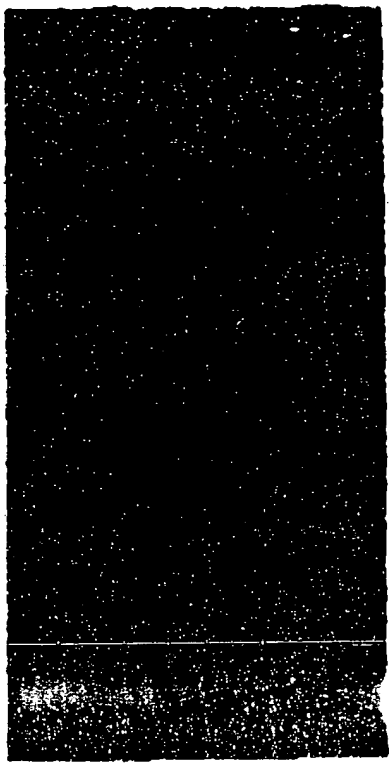
Supernatant LPS. The release of LPS (O-antigen) into culture supernatants by *S. typhimurium* G-30, as measured by radioimmunoassay, increased as the exogenous galactose concentration increased over the range 0.05 to 0.5 mM (Fig. 8). The small amounts of O-antigen released by cells growing without added galactose may have been derived from traces of galactose present in PPBR (21) or from minor leakiness of the *galE* mutation in this strain. To assure uniform incorporation of [^{3}H]galactose (present at a concentration of 0.7 μM) while allowing accumulation of sufficient amounts of supernatant LPS for subsequent analysis, a concentration of nonradioactive galactose of 0.1 mM was arbitrarily chosen for the labeling experiments. Under these conditions, supernatant LPS contained 0.6 to 1.4% of the radioactivity incorporated into cells from the same cultures (four determinations). Similarly, strain PR 122 cells were labeled with [^{3}H]glucosamine in the presence of 0.08 mM nonradioactive D-glucosamine and 0.06 mM D-galactose. Supernatant LPS con-

FIG. 6. *Gradient SDS-PAGE of strain PR 122 (galE nag) labeled with N-acetyl-[^{3}H]glucosamine or [^{3}H]galactose. The orientation of the gel was the same as in Fig. 4 and 5. Lane 1 contained outer membranes of PR 122 labeled with N-acetyl-[^{3}H]glucosamine in the absence of exogenous galactose; 250,000 cpm was loaded. Lane 2 contained 25,000 cpm of the same preparation. Lane 3 contained 250,000 cpm of N-acetyl-[^{3}H]glucosamine-labeled outer membrane from strain PR 122 cells grown in the presence of 0.08 mM nonradioactive D-galactose. Lane 4 contained 250,000 cpm of [^{3}H]galactose-labeled outer membranes from cells grown in the presence of 0.08 mM nonradioactive D-galactose. The heavy band at the top of lanes 1, 2, and 3 represents labeled peptidoglycan. In the absence of exogenous galactose (lanes 1 and 2), radioactive glucosamine was found in a single band at a migration position consistent with that of Rc LPS (in lane 1, the minor bands probably represent larger LPS molecules synthesized from traces of galactose in the medium). When exogenous galactose was present, R-core and O-chains were synthesized, and the distribution of radioactive glucosamine reflected the number of molecules of each LPS size species (lane 3); only traces were present in the upper parts of the gel corresponding to peaks I and II. When cells were labeled with radioactive galactose, the distribution of radioactivity reflected the presence of galactose in both R-core and O-chain repeat units (lane 4) and was identical to the distribution of radioactivity found with galactose-labeled strain G-30.*

tained approximately 0.5% of the radioactivity incorporated into cells.

Sucrose gradient ultracentrifugation of radiolabeled supernatant LPS revealed a single peak of ^{3}H activity. The peak density varied with different preparations, possibly because of differences in the aggregation of membrane fragments with media constituents, but it was never more than 1.20 g/ml.

Comparison of outer membrane and supernatant LPS compositions. Since the structure of lipid A–R-core does not vary with the addition of O-chain repeat units, the extent of labeling of strain PR 122 LPS with *N*-acetyl-[^{3}H]glucosamine should provide an estimate of the number of LPS molecules in each size category. A comparison of the supernatant fragments and outer membranes which were radiolabeled with *N*-acetyl-[^{3}H]glucosamine did not demonstrate a consistent difference in the distribution of radioactivity into peaks II and III (tube SDS-PAGE, Table 1). It was not possible to measure the radioactivity incorporated into peak I because of the contamination of this peak in outer membrane gels with glucosamine-labeled peptidoglycan (Fig. 3C); and the number of counts in peak II was low. Thus, we used strain G-30 labeled with [^{3}H]galactose for a similar analysis (Table 2). This approach suggested that supernatant fragments were slightly enriched in the amounts of radioactivity incorporated into long-O-chain LPS (peaks I and II) relative to short-O-chain LPS (peak III). The direction of the difference was consistent (in each of five comparisons, supernatant fragments had a greater percentage of counts in peak I and II LPS; $P = 0.031$ using a one-tailed Wilcoxon signed-rank test). However, the quantitative differences were small and variable (1.75 to 11.25%). Moreover, galactose labeling emphasized the quantitative influence of a small number of molecules (those which contained many O-chain repeat units). In both of the above-described experiments, an inspection of the tube gel profiles did not reveal subtle differences in the compositions of peak III in supernatant fragment and outer membrane LPS, although such differences were suggested by slab SDS-PAGE of these preparations (Fig. 7). We conclude that supernatant LPS is probably enriched in molecules with long O-chains, although the number



1 2 3 4 5

FIG. 7. *SDS-PAGE comparison of outer membrane and supernatant LPS. Preparations from two separate experiments are shown. Lanes 1, 2, and 3 contained outer membranes, supernatant blebs, and phenol-water-extracted LPS, respectively, from S. typhimurium G-30 grown with [^{3}H]galactose as described in the text; 20,000 cpm of each preparation was loaded. Lanes 4 and 5 contained outer membrane and supernatant LPS, respectively, from a different experiment; 22,000 cpm of each preparation was loaded. For fluorography, the prepared gel was exposed to X-ray film for 3 weeks. In each experiment, supernatant LPS appeared somewhat depleted in LPS with short O-chains and enriched in LPS with long O-chains.*

of molecules which accounts for this difference is small.

DISCUSSION

LPS synthesis in *S. typhimurium* has been investigated intensively (22, 24). The R-core is made on the inner membrane by stepwise addition of sugars to ketodeoxyoctonate-lipid A. Synthesis of O-polysaccharide occurs independently of lipid A-R-core synthesis; oligosaccharide repeat units, formed on C_{55} polyisoprenoid lipid carriers, are polymerized on the inner membrane

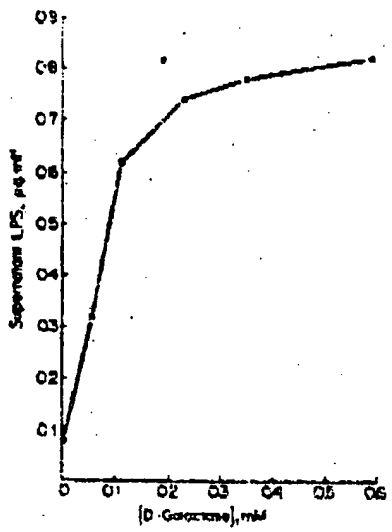


FIG. 8. *Accumulation of LPS (O-antigen) in culture supernatants of S. typhimurium G-30. Strain G-30 cells were grown in 20-ml PPBE cultures containing different concentrations of D-galactose. The growth conditions, preparation of supernatants, and LPS assay were as described in the text. Growth was stopped at a cell density of 6 × 10^8 viable cells per ml. Each point is the average of two determinations. Accumulation of O-antigen in the culture supernatant was dependent upon D-galactose concentration over the range studied.*

TABLE 1. *LPS size distribution in outer membranes and supernatant blebs of S. typhimurium PR 122*[a]

Expt	Sample	Gel	cpm of *N*-acetyl-[^{3}H]glucosamine		Total cpm	% of long-O-chain LPS[b]
			Peak II	Peak III		
1	S	a	1,984	26,184	28,148	7.0
		b	1,200	22,893	24,299	4.9
	OM	a	7,474	116,348	123,822	6.0
		b	8,728	99,589	108,589	8.4
2	S	a	1,394	20,988	22,879	6.2
		b	878	21,409	22,287	4.4
	OM	a	9,074	136,338	145,412	6.2
		b	7,645	135,236	142,880	5.4

[a] Cells were labeled with *N*-acetyl-[^{3}H]glucosamine as described in the text. Outer membrane (OM) and supernatant (S) fractions from peaks of ^{3}H activity on sucrose gradients were analyzed by tube SDS-PAGE. Samples from a given experiment were electrophoresed together. The counts in each peak were summed and corrected for background counts; peak III included all counts below the downslope of peak II (in the gel shown in Fig. 8A, slices 21 through 47).

[b] (Counts per minute in peak II/total counts per minute) × 100.

TABLE 2. *LPS size distribution in outer membranes and supernatant blebs of S. typhimurium C-30*[a]

Expt	Sample	Gel	cpm of [^{3}H]galactose		Total cpm	% Of long-O-chain LPS[b]	% Difference (S minus OM)
			Peaks I and II	Peak III			
1	S	a	1,339	687	2,026	66.1	
		b	1,778	683	2,461	72.2	
	OM	a	86,509	51,909	138,418	62.4	6.35
		b	53,490	31,271	84,761	63.1	
2	S	a	13,641	5,240	18,881	72.2	
		b	17,722	6,490	24,212	73.1	
	OM	a	157,262	81,246	238,508	65.9	8.40
		b	168,134	100,517	268,651	62.6	
3	S	a	4,941	2,226	7,167	69.0	
		b	8,275	3,650	11,925	69.4	
	OM	a	25,472	18,165	43,637	58.4	11.26
		b	54,776	40,506	95,284	57.5	
4	S	a	5,866	2,520	8,386	70.0	
		b	5,103	2,187	7,290	70.0	
	OM	a	33,824	17,896	51,720	65.4	3.95
		b	33,200	16,600	49,800	66.7	
5	S	a	4,050	1,858	5,917	68.6	
		b	3,661	2,422	6,083	60.1	
	OM	a	26,676	15,586	42,264	63.1	1.76
		b	28,993	17,710	46,703	62.1	

[a] Cells were labeled with [^{3}H]galactose as described in the text. For details, see Table 1, footnote a. The distribution of counts into peaks I and II varied from experiment to experiment; for simplicity, these counts were summed and compared with the counts in peak III. S, Supernatant; OM, outer membrane.

[b] (Counts per minute in peaks I and II/total counts per minute) × 100.

into O-chains. Transfer of the completed O-side chain to the R-core (the ligase reaction) is followed by translocation of the completed LPS to the outer membrane. Translocation is irreversible but somewhat nonspecific, since R-core LPS, as well as complete (O-antigen-containing) LPS, may be transferred. Once core LPS molecules are present in the outer membrane, they are not "completed" by the addition of O-side chains (19, 25). Translocation appears to involve membrane sites which overlie zones of adhesion between the inner and outer membranes (19).

Heterogeneity is known to occur at several of the steps in *Salmonella* LPS synthesis, including polymerization of O-chain repeat units (22, 27). Previous workers have analyzed the lengths of O-polysaccharide chains by using gel filtration (9) or methods (e.g., methylation) which determine the number of end groups in a population of isolated polysaccharide molecules (12, 13, 23). Estimates of the average *S. typhimurium* O-chain length in a variety of strains have ranged from 4 (13, 23) to 8 to 10 (13) repeat units. These methods, which require isolation and purification of the O-polysaccharide before analysis, do not identify small differences in O-chain length within a large population of molecules. In contrast, the SDS-PAGE gradient method described here separates LPS molecules differing in as few as two or three saccharides (as observed for *S. minnesota* R-core LPS [Fig. 4A]), and it can be used to analyze the LPS in a variety of starting materials, labeled or unlabeled. This method should be useful for further study of LPS synthesis in vivo, as it allows simple resolution of LPS molecules of different sizes (2). Similar conclusions concerning LPS size heterogeneity have been reached recently by workers using discontinuous (Laemmli) gel systems (7, 27). Our results differ from the results of these investigators in two ways. First, we did not find the doublet bands which were present in the discontinuous gels. These authors considered several explanations for these doublets, which seemed to differ in composition depending upon conditions of LPS storage (7). Our results sug-

gest that a comparison of the two gel systems in which the same preparations are used may help solve this problem. Second, our gels were suitable for staining with periodic acid-Schiff reagent. Although this method gave poorer resolution than autoradiography or fluorography, bands were sufficiently distinct to allow study of nonradioactive preparations.

Our analysis indicates that, under the growth conditions used in these experiments, *S. typhimurium* makes LPS molecules of many sizes. LPS with short O-chains appear to be synthesized preferentially, at least in the mutants studied. The striking size heterogeneity was not anticipated. Among the possible explanations are (i) synthesis of different O-chain lengths at different phases of growth or in different regions of the cytoplasmic membrane, (ii) nonselective polymerase or ligase or both and (iii) synthesis of different O-chain lengths by different cells in the culture (27). Although our data do not distinguish among these possibilities, we also found size heterogeneity in LPS from a clinical isolate of *S. typhimurium*; thus, it is unlikely that this phenomenon is limited to the mutants used for the present experiments.

Several studies of outer membrane assembly have used antibodies to O-antigen to identify newly synthesized LPS in uridine diphosphate-galactose-4-epimerase-deficient mutants of *S. typhimurium* (18, 19). Newly synthesized LPS appeared on bacterial surfaces in patches or clusters which were adjacent to apparent zones of adhesion between inner and outer membranes. Our estimates mentioned above, made by using similar strains, indicate that molecules with long O-chains constitute a minority of outer membrane LPS. Since long-O-chain LPS offer more antigenic sites for antibody binding than short-O-chain LPS, the observed surface patches may not indicate bulk LPS translocation at these sites, but rather translocation sites for LPS with long O-chains. In any case, the size heterogeneity of LPS in outer membranes indicates that these bacteria translocate LPS molecules of many sizes from inner membranes to outer membranes. The translocation mechanism(s) thus may be multiple or to have little size specificity.

There is evidence that supernatant blebs arise from specific outer membrane sites; as suggested by Witholt et al. for *E. coli*, possible sites of origin for supernatant blebs include membranes rendered redundant at septum formation (cell division), areas of membrane deficient in murein-lipoprotein, and sites of insertion of new LPS into outer membranes (8, 17). We anticipated that the supernatant fragments might be enriched in LPS molecules with long O-chains, since the long hydrophilic polysaccharides might facilitate the release of LPS from the outer membrane into the aqueous environment. We compared uter membrane and supernatant fragment LPS in two *S. typhimurium* strains. The results of these experiments are consistent with the conclusion that supernatant LPS are probably enriched in long-O-chain LPS, although the number of molecules which accounts for this difference is small. It should also be noted that strains with only core LPS structures shed membrane fragments (1). Indeed, it seems that *S. typhimurium* may shed membrane fragments by more than one mechanism (or from more than one site); the supernatant fragments studied here would then be a mixture of such fragments, and the LPS compositions observed would reflect the contribution of different components of the mixture. Our attempts to separate such components by density gradient ultracentrifugation were not successful, however.

These findings may be important for an understanding of the pathogenesis of infections caused by this bacterium and other gram-negative bacteria. Both outer membrane and supernatant membrane blebs contain lipid A, the toxic moiety of LPS. Since O-polysaccharides extend from the bacterial surface into the surrounding environment, the observed heterogeneity of O-chain lengths suggests that the surface topography of these cells may be quite irregular and that the accessibility (surface exposure) of the lipid A moiety probably differs for different surface regions or for different cells. It is reasonable to expect that these differences may influence the biological activity of LPS by modulating host-lipid A interactions.

ACKNOWLEDGMENTS

We thank Leon Rifkin and Emil Gotschlich for careful and critical review of the manuscript and Joan Reisch for statistical advice. Fred Neidlet, G. Bryan Holland, C. Gomez-Sanchez, and Thomas Smith generously provided equipment for the experiments.

This work was supported by Public Health Service Young Investigator Grant AI-14942 from the National Institute of Allergy and Infectious Diseases (R. M.) and Uniformed Services University of Health Sciences grant RG7302 (P. R.).

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